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Isoprotein analysis by ion-exchange chromatography using a linear pH gradient combined with a salt gradient

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ABSTRACT

Isoproteins of human monoclonal antibodies with a p*I* range between 8.45 and 8.70 or 8.15 and 8.65 were separated by ion-exchange chromatography with a linear ascending pH gradient combined with a linear descending salt gradient using borax, mannitol and salt. The isoproteins were eluted according to their isoelectric points as demonstrated by conventional isoelectric focusing. Preparative purification and monitoring of the isoprotein composition of human monoclonal antibodies during a purification process is also presented to demonstrate the applicability of the method.

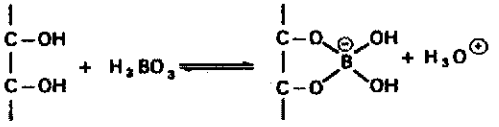
INTRODUCTION

Owing to post-translatorial modifications, monoclonal antibodies exhibit strong micro-heterogeneity [1]. Several methods, such as isoelectric focusing (IEF) [2,3], chromatofocusing (CF) [4], hydrophobic interaction chromatography (HIC) [5] and capillary zone electrophoresis (CZE) [6], have been described for the analysis and preparation of their isoforms. Isoelectric focusing is mainly used for the analytical determination of isoform patterns [7], whereas HIC and CF are the methods of choice for preparative purposes.

Ion-exchange chromatography is a powerful tool for the purification of monoclonal antibodies. In most applications step gradients are used to accomplish elution [8]. In principle, ion-exchange chromatography can also be used for separating the various isoforms of monoclonal antibodies. By generating a linear pH gradient the isoforms can easily be eluted near their isoelectric points. Shukun *et al.* [9] used borax–mannitol solution in free-flow electrophoresis for generating a pH gradient. The addition of mannitol to borate buffer [10] changes the pH to an acidic milieu. The low conductivity of this buffer allows the use for ion-exchange chromatography. In this work, linear pH–salt gradients were used for the analytical and preparative separation of isoproteins. As model proteins human monoclonal antibodies were investigated.

THEORY

A linear pH gradient is achieved by the reaction of the *cis*-diol of mannitol with borate, which liberates H_3O^+ in a stoichiometric manner:



A stable complex is formed, which does not undergo ageing. The theory of the formation of new acids by association of boric acids and planar diols is due to the fact that the "ionization

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constants" of such acids often obey an equation of the form

$$K^{**} = C_{2n}K_1K_n + K_1$$

The "ionization constant" K^{**} varies with the concentration of the ligand C_{2n} and is independent of the concentration of the boric acid at concentrations below 0.1 *M*. K_n is the association constant of the boric acid and the diol and K_1 is the first ionization constant of boric acid.

To generate a linear pH gradient for ion-exchange chromatography, a strong ion exchanger must be used. The dissociation constant of the functional group is not significantly affected by the pH of the mobile phase [11]. This assumption is valid for a working pH range between 4 and 9. Therefore, the continuous pH change in the mobile phase is not affected by ionizable groups of the ion exchanger. In contrast to chromatofocusing, the ion exchanger is not involved in the formation of the pH gradient.

EXPERIMENTAL

Model proteins

Human monoclonal antibodies [12] were purified as described in detail by Unterluggauer *et al.* [13]. Briefly, the clarified culture supernatant was concentrated by ultrafiltration, desalted by diafiltration and chromatographed on protein A Sepharose fast flow and S-Sepharose fast flow and desalted with Sephadex G-25 coarse. Both antibodies, "3D6" and "Virgil", belong to isotype IgG1. "3D6" has an isoelectric range between p*I* 8.45 and 8.70 and "Virgil" between p*I* 8.15 and 8.65.

Ion-exchange chromatography

Mono S HR5/5 and Mono S HR16/10 columns (Pharmacia, Uppsala, Sweden) were used. The columns were connected to a fast protein liquid chromatographic system. UV absorbance at 280 nm, pH and conductivity were monitored continuously. For elution a linear pH gradient was generated by mixing the starting buffer consisting of 5 m*M* borate, 45 m*M* mannitol and different concentrations of NaCl (depending on the binding behaviour of the model proteins) with 5 m*M* borate.

Isoelectric focusing

Isoelectric focusing was performed with carrier ampholytes in the pH range 3–10 according to Westermeier [14]. The gel was developed by

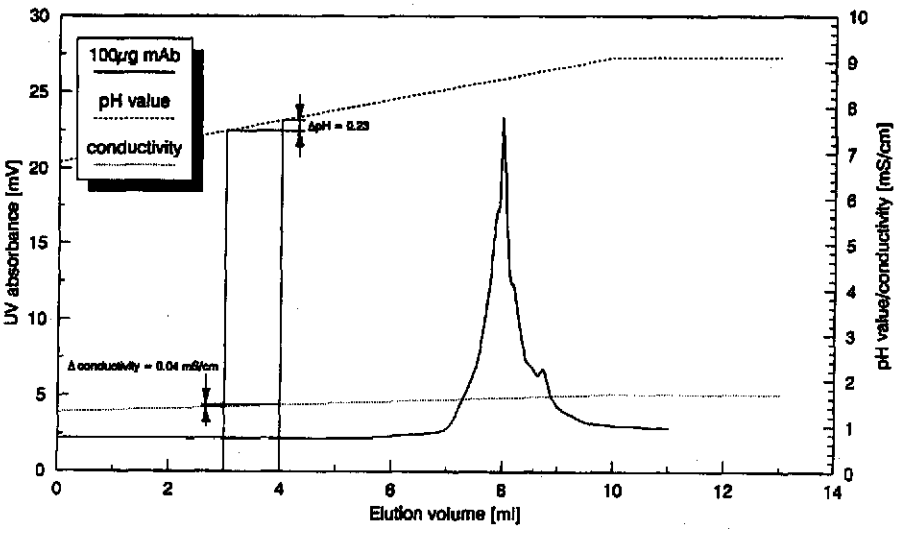

Fig. 1. Separation of isoproteins of human monoclonal antibody "3D6" by ion-exchange chromatography on a Mono S column (HR 5/5); column volume (v_t) = 1 ml; 5 cm × 0.5 cm I.D. Sample, 100 µg of monoclonal antibody dissolved in 25 µl of starting buffer and loaded on a 1-ml column; starting buffer, 10 m*M* borate, 70 m*M* mannitol (pH 6.8), κ = 1.3 mS/cm; elution buffer, 10 m*M* borate (pH 9.1), κ = 1.7 mS/cm. Elution was effected with a linear pH gradient over ten column volumes at a flow-rate of 0.1 ml/min (30.6 cm/h).

silver staining according to Heukeshoven and Dernick [15].

Chromatography data acquisition

UV absorbance, pH and conductivity signals were processed on a Nelson Analytical (Cupertino, CA, USA) chromatography data system.

RESULTS

Two human monoclonal antibodies with p*I* ranges between 8.45 and 8.70 and between 8.15 and 8.65 were used as model proteins. To resolve the isoproteins with linear pH gradients an elution procedure analogous to chromatofocusing was designed. The sample was transferred into the starting buffer by gel permeation on Sephadex G-25 in PD-10 columns (Pharmacia). Elution was performed with a linear gradient from pH 7.0 to 9.1.

Development of the linear gradient

The antibody "3D6" with p*I* 8.45–8.70 was used as model antibody for the development of a linear gradient. To improve the resolution the following experiments were carried out. To increase the insufficient resolution obtained with a linear pH gradient (Fig. 1), salt was added to the starting buffer in an amount that still allowed binding of the antibody to Mono S. A combined ascending pH and a descending salt gradient (Fig. 2) was generated by this procedure. However, the resolution was still unacceptable. A steeper salt gradient was generated by decreasing the borax concentration from 10 to 5 m*M* to obtain a conductivity equal to the conditions described in Fig. 2. The results of this run are shown in Fig. 3. Subsequently optimum resolution was achieved when the gradient volume was doubled (Fig. 4).

Preparative purification

For preparative purification of the various isoforms, the human monoclonal antibody "Virgil" of p*I* 8.15–8.65 was used. Prior to scale-up, the resolution was optimized with a 1-ml column applying the same conditions as developed for antibody "3D6" (Fig. 5). The preparative purification was carried out on a Mono S HR16/10 column with a total column volume of 22 ml. The partially separated isoforms were collected in fractions (Fig. 6) and analysed by IEF (Fig. 7)

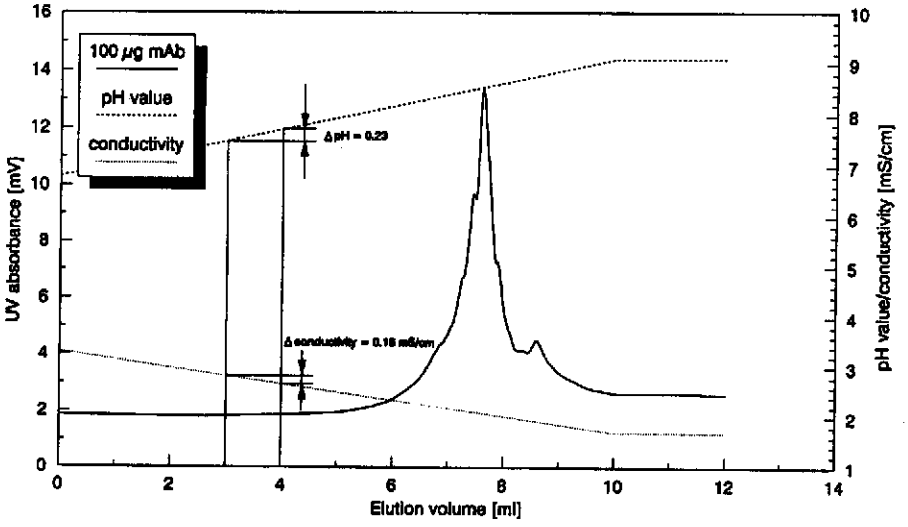

Fig. 2. Separation of isoproteins of human monoclonal antibody "3D6" by ion-exchange chromatography on a Mono S column (HR 5/5). Sample, 100 µg of monoclonal antibody dissolved in 25 µl of starting buffer and loaded on a 1-ml column; starting buffer, 10 m*M* borate, 70 m*M* mannitol, NaCl (pH 6.8), $\kappa = 3.3$ mS/cm; elution buffer, 10 m*M* borate (pH 9.1), $\kappa = 1.7$ mS/cm. Elution was effected with a linear pH–salt gradient over ten column volumes at a flow-rate of 0.1 ml/min. Other conditions as in Fig. 1.

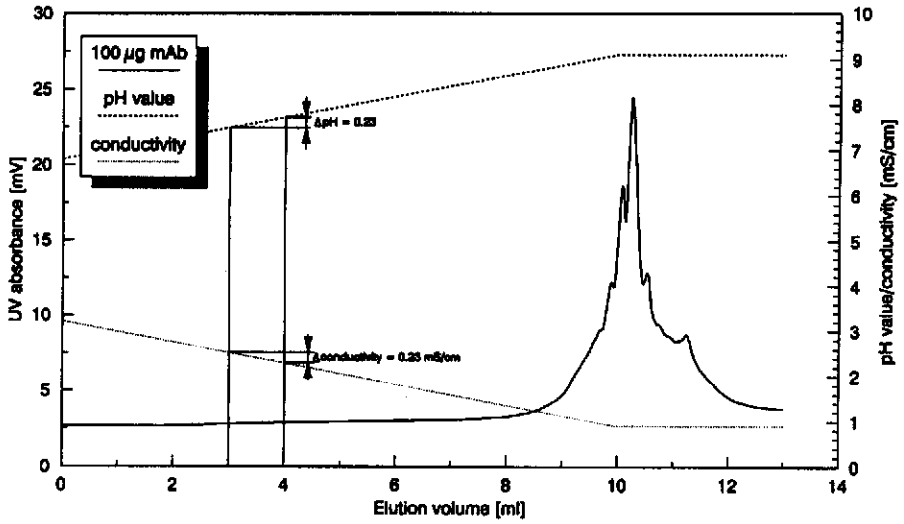

Fig. 3. Separation of isoproteins of human monoclonal antibody "3D6" by ion-exchange chromatography on a Mono S column (HR 5/5). Sample, 100 μg of monoclonal antibody dissolved in 25 μl of starting buffer and loaded on a 1-ml column; starting buffer, 5 mM borate, 40 mM mannitol, NaCl (pH 6.8), $\kappa = 3.2$ mS/cm; elution buffer, 5 mM borate (pH 9.1), $\kappa = 0.9$ mS/cm. Elution was effected with a linear pH–salt gradient over ten column volumes at a flow-rate of 0.1 ml/min. Other conditions as in Fig. 1.

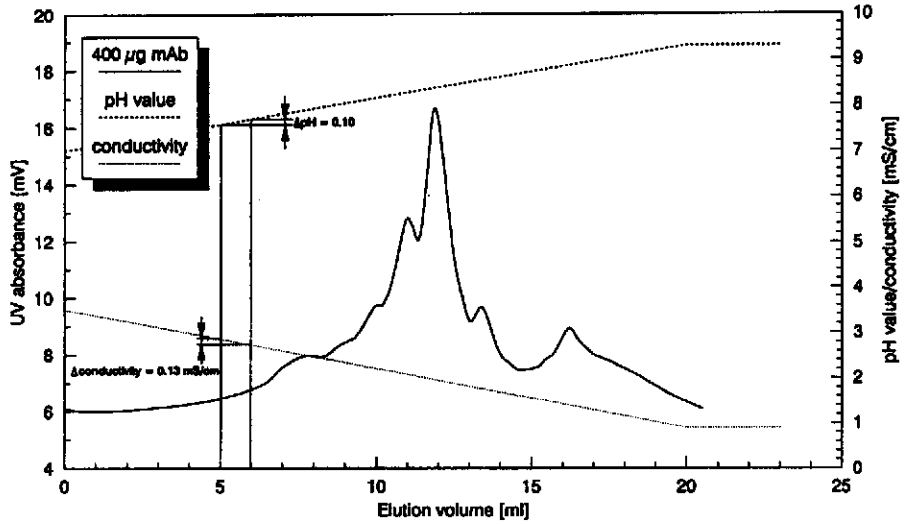

Fig. 4. Separation of isoproteins of human monoclonal antibody "3D6" by ion-exchange chromatography on a Mono S column (HR 5/5). Sample, 400 μg of monoclonal antibody dissolved in 100 μl of starting buffer and loaded on a 1-ml column; starting buffer, 5 mM borate, 40 mM mannitol, NaCl (pH 7.1), $\kappa = 3.5$ mS/cm; elution buffer, 5 mM borate (pH 9.1), $\kappa = 0.9$ mS/cm. Elution was effected with a linear pH–salt gradient over twenty column volumes at a flow-rate of 0.2 ml/min (61.1 cm/h). Other conditions as in Fig. 1.

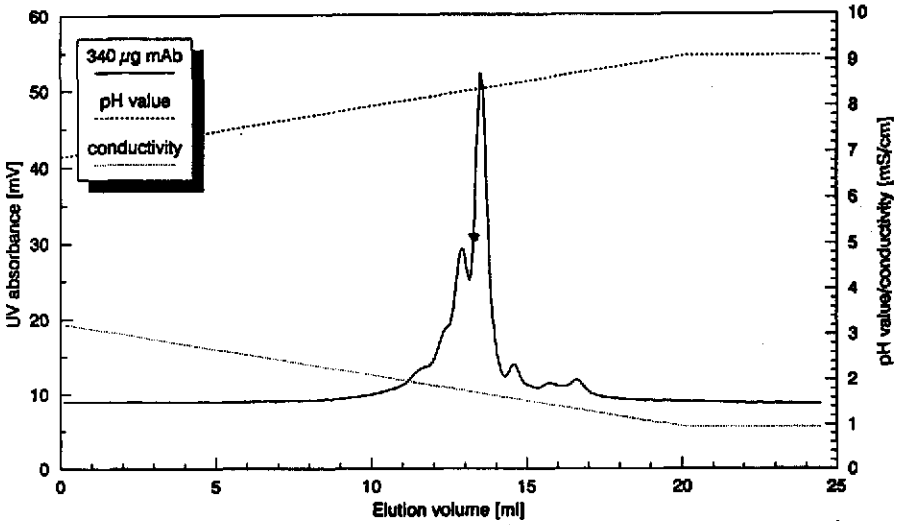

Fig. 5. Analytical separation of isoproteins of human monoclonal antibody "Virgil" by ion-exchange chromatography on a Mono S column (HR 5/5). Sample, 340 µg of monoclonal antibody dissolved in 31.0 ml of starting buffer and loaded on a 1-ml column; starting buffer, 5 m*M* borate, 45 m*M* mannitol, 0 m*M* NaCl (pH 6.9), $\kappa = 3.2$ mS/cm; elution buffer, 5 m*M* borate (pH 9.1), $\kappa = 0.9$ mS/cm. Elution was effected with a linear pH–salt gradient over twenty column volumes at a flow-rate of 0.2 ml/min. Other conditions as in Fig. 4.

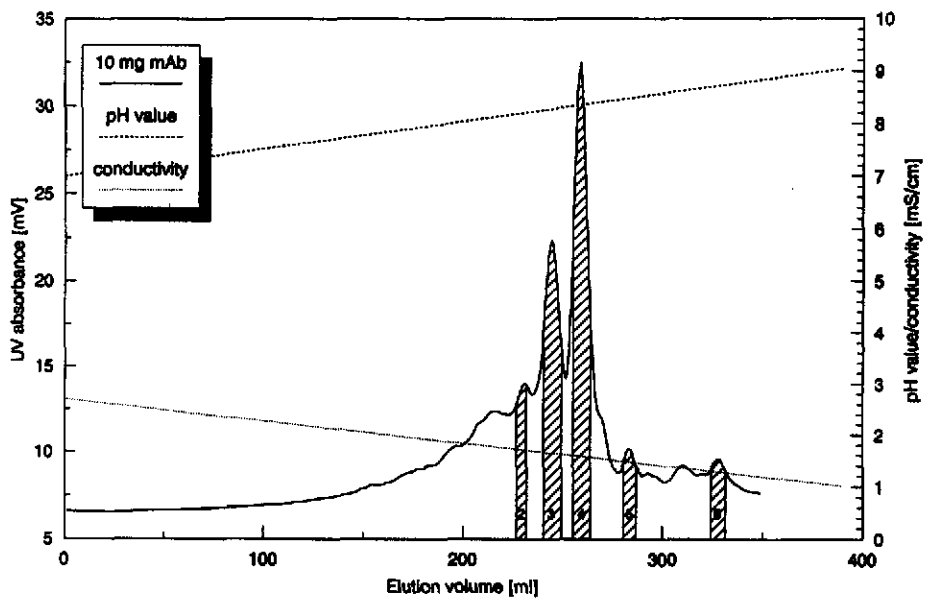

Fig. 6. Preparative separation of isoproteins of human monoclonal antibody "Virgil" by ion-exchange chromatography on a Mono S column (HR 10/16). Sample, 10 mg of monoclonal antibody dissolved in 23.0 ml of starting buffer and loaded on a 20-ml column; starting buffer, 5 m*M* borate, 45 m*M* mannitol, 20 m*M* NaCl (pH 7.0), $\kappa = 2.8$ mS/cm; elution buffer, 5 m*M* borate (pH 9.1), $\kappa = 0.9$ mS/cm. Elution was effected with a linear pH–salt gradient over twenty column volumes at a flow-rate of 2.0 ml/min.

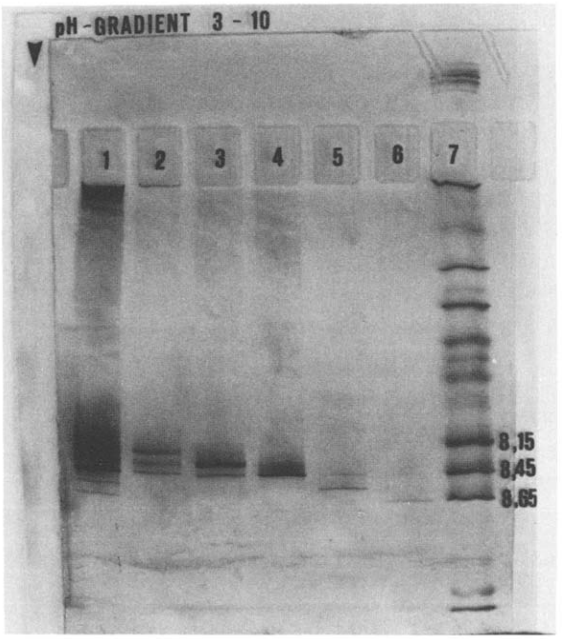

Fig. 7. Isoelectric focusing of the fractions in Fig. 6. Samples: lane 1 = starting material (human monoclonal antibody "Virgil"); lanes 2–6 = isoprotein fractions; lane 7 = p*I* 3–10 marker.

to demonstrate that the isoforms elute according to their isoelectric points. Aliquots of the collected fractions were desalted on Sephadex G-25 in PD-10 columns and rechromatographed on Mono S HR5/5 (Fig. 8).

Process monitoring

This method was also used for monitoring the purification process of human monoclonal antibody "Virgil". The antibody was produced by animal cell culture using RPMI medium containing 2% foetal calf serum.

The isoform pattern in the particular purification steps of "Virgil" is demonstrated in Fig. 9A and B. Peak areas were integrated by a Nelson chromatography data system using force dropline integration and a project horizontal baseline rearward setting. The area of the most dominant isoprotein was arbitrarily defined as 100%, and peak areas of the other isoproteins were expressed as a percentage relative to this. The experiments showed that the purification step with protein A Sepharose fast flow causes a loss of the most basic isoform (Table I).

TABLE I

QUANTITATIVE ISOFORM DISTRIBUTION OF HUMAN MONOCLONAL ANTIBODY ("VIRGIL") DURING A PURIFICATION PROCESS SHOWING A LOSS IN AMOUNT OF ISOPROTEIN 4 AFTER PROTEIN A SEPHAROSE

The samples of single antibody purification sequence were analysed in duplicate.

Sample	Area (%)			
	Peak 1	Peak 2	Peak 3	Peak 4
Diaretentate 1	41.0	100	10.0	17.1
Diaretentate 2	42.2	100	7.8	16.2
Eluate protein A Sepharose 1	45.4	100	9.8	4.9
Eluate protein A Sepharose 2	41.9	100	8.2	4.4
Eluate S-Sepharose 1	43.2	100	8.7	3.7
Eluate S-Sepharose 2	45.6	100	9.9	4.2

DISCUSSION

A linear pH gradient of 20 column volumes formed by mixing 5 m*M* borate, 45 m*M* mannitol and 20 m*M* NaCl with 5 m*M* borate gives sufficient resolution on Mono S for separating the isoproteins of human monoclonal antibodies with a p*I* range between 8.45 and 8.70 or 8.15 and 8.65. Identical resolutions were obtained on 1-ml and 22-ml columns (Figs. 5 and 6). Further, ascending pH gradients in combination with descending salt gradients can improve the resolution of isoprotein separation (compare Figs. 1 and 4). The various isoforms are resolved according to their isoelectric points as demonstrated by IEF (Fig. 7). The antibodies elute in order of their p*I* values. Isoproteins are very homologous with each other, differing only in several charged carbohydrate moieties. These moieties are at the surface of the protein and elution in the ion-exchange mode is coincidental with the p*I* values. The oligosaccharide might also form a complex with borate, like mannitol and borax in the mobile phase. Such complex

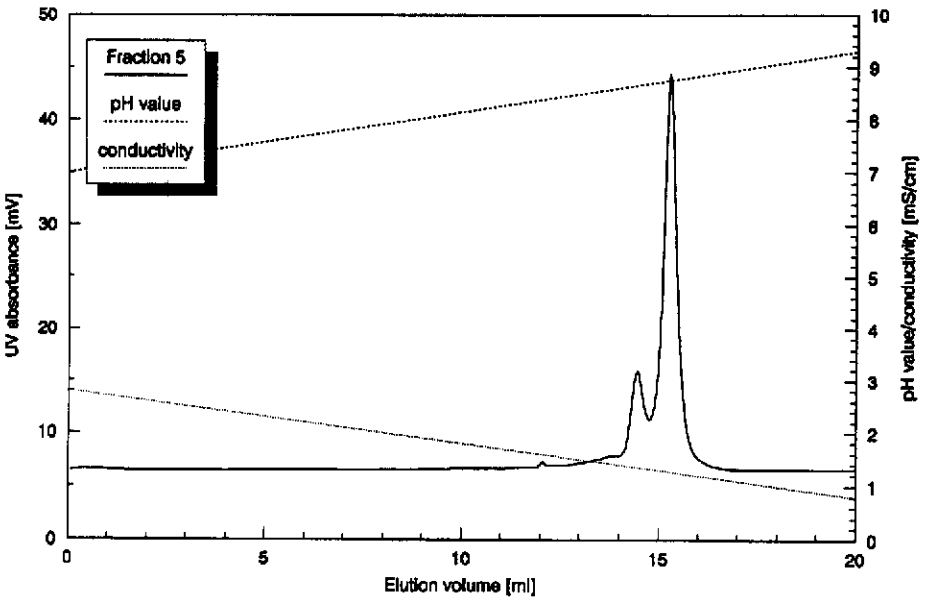

Fig. 8. Rechromatography of separated isoproteins of "Virgil" by ion-exchange chromatography on a Mono S column (HR 5/5). Sample, fraction 5 from Fig. 6 dissolved in 21.0 ml of starting buffer and loaded on a 1-ml column; starting buffer, 5 m*M* borax, 45 m*M* mannitol, 20 m*M* NaCl (pH 7.0), $\kappa = 2.8$ mS/cm; elution buffer, 5 m*M* borax (pH 9.1), $\kappa = 0.9$ mS/cm. Elution was effected with a linear pH–salt gradient over twenty column volumes at a flow-rate of 0.2 ml/min.

formation may contribute to the separation. Additional charges might be introduced into the surface of the antibody molecule. Brena *et al.* [18] also reported the selective adsorption of immunoglobulins on phenylboronate–agarose. An interaction between the sugars from the IgG molecule and H_3BO_3 was confirmed by this observation. Complex formation has also been used for the separation of polyhydroxy compounds such as sugars by ion-exchange chromatography [16]. Further investigations to explain this involvement of complex formation between IgG and H_3BO_3 are in progress. The complex formation between IgG and H_3BO_3 can enhance the separation but the combined pH–salt gradient seems to be the major contributor to the resolution of isoproteins. Without salt, just with the linear pH gradient, the resolution is lower than with the combined pH–salt gradient (compare Figs. 1 and 3).

The advantages of ion-exchange chromatography with linear pH–salt gradients are as follows. The buffer composition (borax, NaCl and mannitol) is simple and inexpensive. After separation the isoforms can be used for immunochemical, *in vivo* and *in vitro* experiments because the problem of ampholine removal is circumvented. In chromatofocusing and conventional IEF, ampholines are needed for the generation of the pH gradients. Ion-exchange chromatography is rapid, in contrast to IEF and CF, but slower than CZE. Compared with IEF, the recovery of isoforms is high. For preparative purposes in IEF the proteins have to be eluted from the polyacrylamide matrix either with a high salt concentration or an electric current. CZE is not scaleable and can therefore only be used for analytical purposes. Ion-exchange chromatography with a pH–salt gradient also allows the quantitative determination of the isoprotein pattern (Table I) in a very accurate manner. IEF fails completely in detecting small quantitative changes in isoform pattern. Coomassie Brilliant Blue is too insensitive to detect these small changes. Unlike Coomassie Brilliant Blue dye, which binds stoichiometrically to most proteins, the silver staining method results in bands which show much greater variation [17].

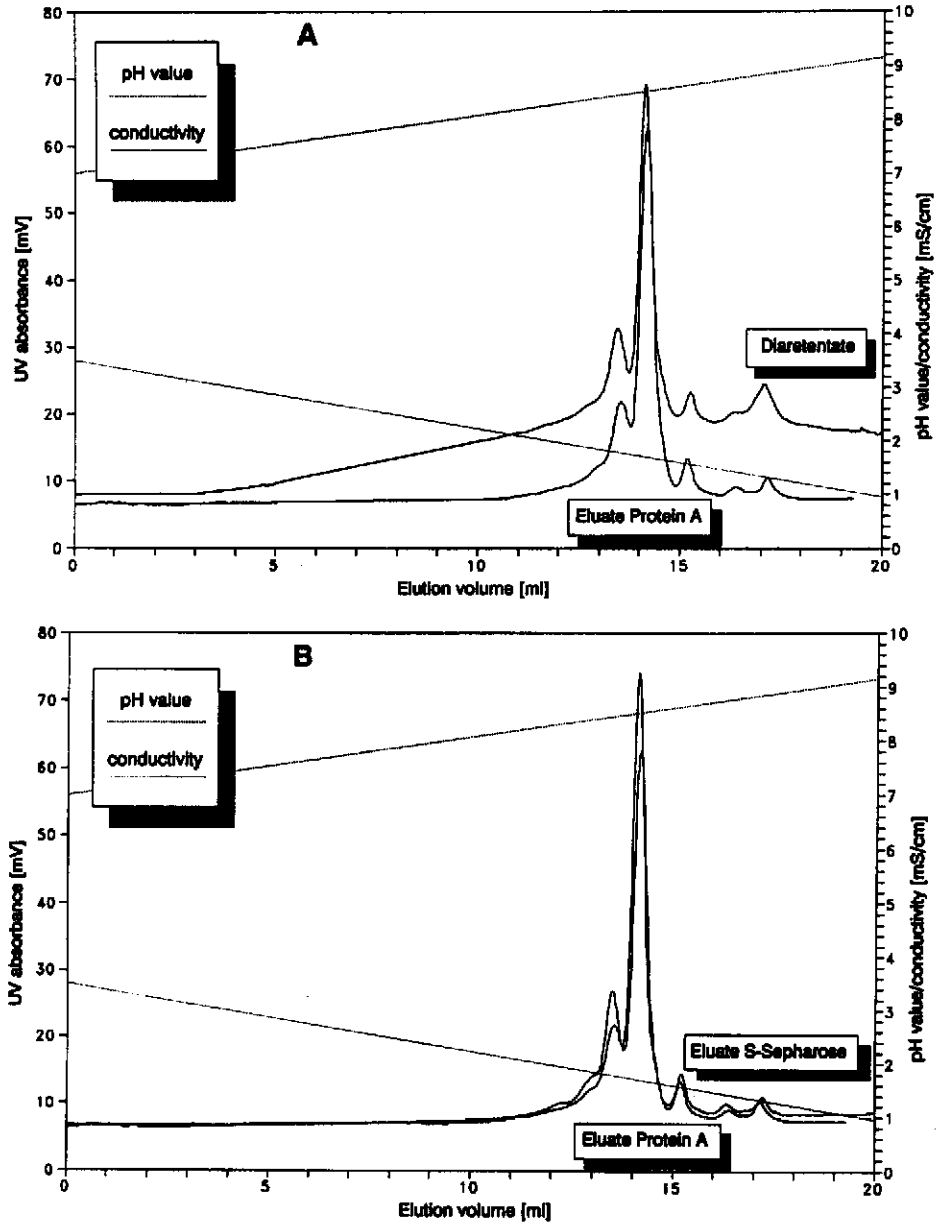

Fig. 9. Comparison of isoform pattern during a purification process of human monoclonal antibody "Virgil" by ion-exchange chromatography on a Mono S column (HR 5/5). Samples: (A) diaretentate and eluate of protein A Sepharose fast flow; (B) protein A Sepharose fast flow and S-Sepharose fast flow. Each sample was diluted to an IgG concentration of 13.5 μg/ml with starting buffer. The sample volume was 20 ml.

The separation of isoforms is essential for the investigation of the heterogeneous nature of native and recombinant proteins and for quality control of recombinant proteins. In this work an attempt was made to demonstrate that ion-exchange chromatography with linear pH–salt gradients could be an alternative method to preparative IEF, CF and CZE. The isoforms of two human monoclonal antibodies could be partially separated according to their isoelectric points. The separation was sufficient to obtain homogeneous samples after rechromatography. Further,

the quantitative determination of isoproteins for process monitoring was possible.

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